Looking beyond death: a morphogenetic role for the TNF signalling pathway

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Summary

Tumour necrosis factor α (TNFα) is a pro-inflammatory mediator with the capacity to induce apoptosis. An integral part of its apoptotic and inflammatory programmes is the control of cell shape through modulation of the cytoskeleton, but it is now becoming apparent that this morphogenetic function of TNF signalling is also employed outside inflammatory responses and is shared by the signalling pathways of other members of the TNF-receptor superfamily. Some proteins that are homologous to the components of the TNF signalling pathway, such as the adaptor TNF-receptor-associated factor 4 and the ectodysplasin A receptor (and its ligand and adaptors), have dedicated morphogenetic roles. The mechanism by which TNF signalling affects cell shape is not yet fully understood, but Rho-family GTPases have a central role. The fact that the components of the TNF signalling pathway are evolutionarily old suggests that an ancestral cassette from unicellular organisms has diversified its functions into partly overlapping morphogenetic, inflammatory and apoptotic roles in multicellular higher organisms.

Key words: Rho, TRAF4, Cell shape, Cytoskeleton

Introduction

Tumour necrosis factor α (TNFα), which was cloned in 1984 (Pennica et al., 1984), was named as such because it can induce tumour regression through the induction of cell death (Carswell et al., 1975). It can bind to two related receptors, TNF receptors 1 and 2 (TNFR1 and TNFR2), which are also used by other, similar ligands. Numerous homologous ligands and receptors have been identified since 1984, and these are classified as members of the TNF and TNFR superfamilies. In humans, the superfamilies currently include more than 40 members, such as lymphotoxin and its receptor CD95L/FasL, TNF-related apoptosis-inducing ligand (TRAIL), nerve growth factor receptor (NGFR), the immune receptor CD40 and its ligand CD40L (Hehlgans and Pfeffer, 2005; Locksley et al., 2001).

In addition to the TNFs and TNFRs, the TNF signalling pathway includes adaptor proteins and signal transducers (reviewed by Aggarwal, 2003; Locksley et al., 2001). Several TNFR-superfamily receptors harbour a death domain in their cytoplasmic tails, which recruits adaptors such as TNF-receptor-associated death-domain protein (TRADD) by dimerization with the adaptor death domain. A complex of signalling proteins including TRADD and TNF-receptor-associated factor 2 (TRAF2) is recruited when TNF binds to TNFR1 (Fig. 1). This leads to diverse downstream events such as the regulation of nuclear factor-κB (NF-κB), Jun N-terminal kinase (JNK) and caspases, which in turn influence cell-death and cell-survival decisions. Other members of the receptor superfamily may use different molecular activation mechanisms and downstream components, and not all of the adaptors are restricted to functions downstream of TNFα and its superfamily members. For example, TRADD and TRAFs are also involved in Toll-like receptor signalling (Bishop and Xie, 2007; Chung et al., 2002; Ermolaeva et al., 2008).

The best-characterized functions of TNF are related to apoptosis and inflammation (reviewed by Aggarwal, 2003; Wajant et al., 2003; Wallach et al., 2002), but these are not the only ones. Indeed, as has been shown using various mouse models, the primary role of TNF is not as a 'killer' molecule. Although TNF induces apoptosis in cells that have genetic aberrations or are otherwise compromised, it does not do so in healthy primary cells. Raised TNF levels do not lead to increased cell death (Kontoyiannis et al., 1999), and loss of TNF signalling does not entail abnormalities in cell numbers or tissue size (Pfeffer et al., 1993). Instead, these mouse models revealed that TNF has effects on morphogenetic processes (such as the control of cell movement and cell shape), not only in the context of inflammatory responses but also during developmental events (Fig. 2; Table 1).

In the absence of TNF signalling, secondary lymphoid organs fail to develop properly (Mebius, 2003; Pasparakis et al., 2000; Victoratos et al., 2006), and this is at least partly due to a migration defect in follicular dendritic cells. Some TNF- and TNFR-superfamily members are dedicated purely to morphogenetic and developmental functions, such as ectodermal organ morphogenesis (Headon and Overbeek, 1999; Houghton et al., 2005; Srivastava et al., 2001; Tucker et al., 2000). In addition, some effects of TNF signalling that have been primarily defined in pro-apoptotic signalling may rather be part of the inflammatory repertoire. Several pro-inflammatory changes that are associated with TNFα treatment [such as increased pulmonary or vascular endothelial-cell permeability (Kohno et al., 1993; Koss et al., 2006; Wojciak-Stothard et al., 1998), and neutrophil or fibroblast recruitment to sites of inflammation (Lokuta and Huttenlocher, 2005; Postlethwaite and Seyer, 1990)] and pro-apoptotic effects associated with TNFα treatment [such as osteoblast apoptosis, fibroblast cytolysis or
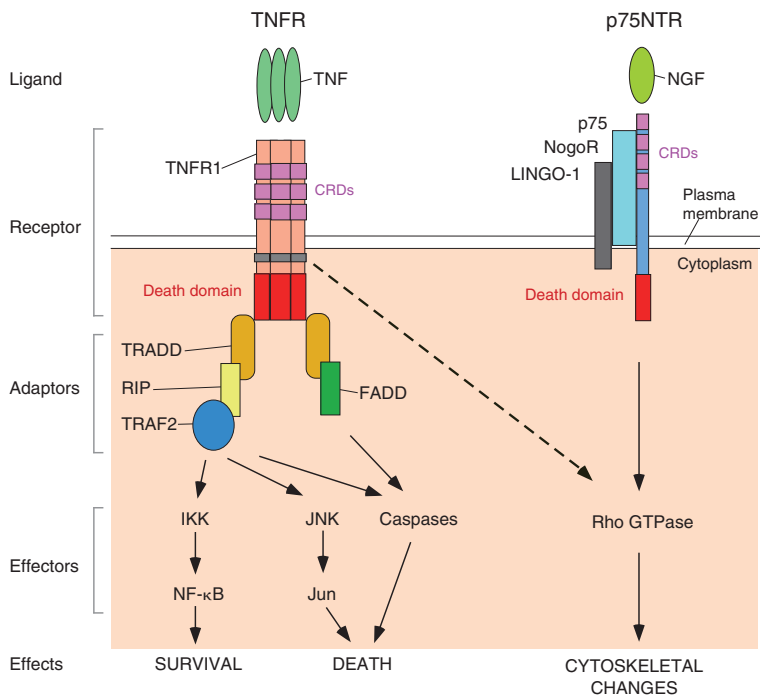
Fig. 1. Components and outcomes of selected TNF-superfamily signalling pathways. (Left) The pathway triggered by TNF is well characterized and the molecules that transmit the signal to the biological outcomes are largely known. In response to binding of TNF, the intracellular part of the TNFR recruits a protein complex containing the TRADD protein, TRAF2 and RIP. TRADD can also interact with FADD protein and caspase-8, leading to caspase-3 activation and apoptosis. TRAF2, RIP and the inhibitor of κB kinase (IKK) function to activate NF-κB, which mediates transcriptional control of cell survival genes. TNF-mediated recruitment of TRAFs is important in activating the JNK cascade. TNF receptor signalling can also have effects that are mediated via Rho family GTPases, as discussed in the text. (Right) The neurotrophin receptor p75 (p75NTR), which is a member of the TNFR-superfamily that is expressed in neurons, forms a heterotrimeric receptor complex with the structurally unrelated NogoR and LINGO-1 proteins. This receptor complex regulates the behaviour of growth cones through the modulation of Rho-family GTPases, which affects the growth-cone cytoskeleton. In members of the TNFR superfamily, the death domain at the receptor cytoplasmic tail mediates interactions with adaptor proteins. A defining feature of the TNFR superfamily is the presence of cysteine-rich domains (CRDs) in the extracellular part.

epithelial-cell apoptosis (Domnina et al., 2002; Domnina et al., 2004; Scanlon et al., 1989; Triplett and Pavalko, 2006)] all involve effects on the cytoskeleton and the cellular machinery regulating the cytoskeleton. The pro-inflammatory effects of TNFα treatment on cultured cells and the associated effects on the cytoskeleton are summarized in Table 1. Recent studies have directly demonstrated effects of TNF signalling on cytoskeletal rearrangements and other morphogenetic processes (Corredor et al., 2003; Haubert et al., 2007; Kutsuna et al., 2004; Lokuta and Huttenlocher, 2005; McKenzie and Ridley, 2007). Thus, cells treated with TNF can be induced to migrate (Postlethwaite and Seyer, 1990) or undergo cell-shape changes that are not normally associated with apoptosis (Puls et al., 1999). In summary, TNF-dependent cell-shape changes occur in

the context of cell death and inflammation but also in situations in which apoptosis and inflammation do not take place.

We discuss here the possible cell-biological mechanisms by which TNF signalling might mediate morphogenetic events. Although many of the crucial questions are still unanswered, it has emerged that Rho GTPases have a major role in morphogenetic signalling, but that there is no strict delineation between the molecular mechanisms that promote cell-shape changes during apoptosis, inflammation or other events downstream of TNF signalling. As many of the same signalling intermediates are used in all cases, the end result of TNF signalling must depend on the balance between various factors, which is probably influenced by crosstalk with other signalling pathways and their input on common mediators such as MAPK, JNK or Rho GTPases, as well as by direct TNFα targets such as NF-κB.
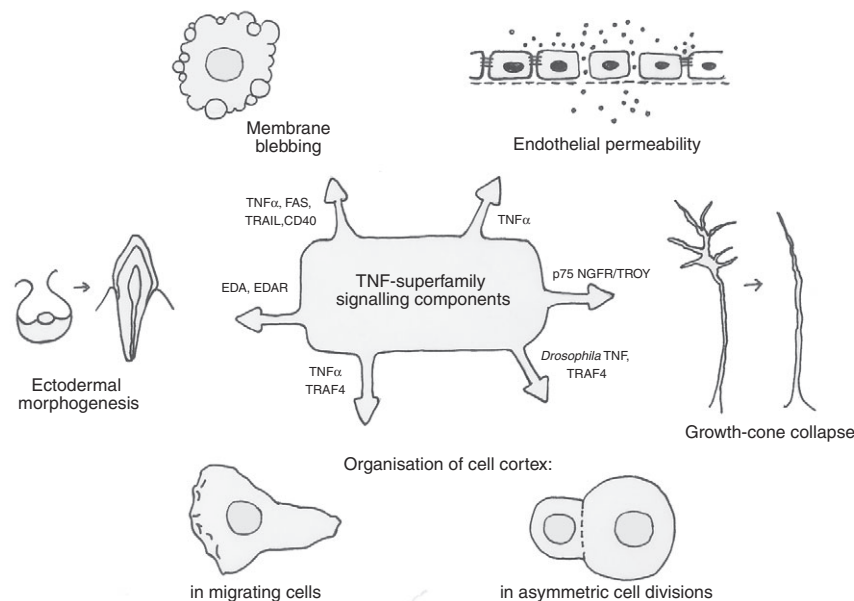
TNFα signals to the actin cytoskeleton

The effects of TNF signalling on the actin cytoskeleton were initially discovered in the context of cell death (Scanlon et al., 1989). Among the most apparent results of TNF-induced apoptosis are drastic changes in cell morphology – such as the rounding-up of the cell and blebbing of the plasma membrane – and the concomitant reorganization of the cytoskeleton. One of the earliest reports on the effect of TNF signalling on actin highlighted the rapid and specific breakdown of stress fibres, which suggested that TNFα might act, at least in part, by directly affecting the reorganization of the actin cytoskeleton (Scanlon

Fig. 2. Examples of the morphogenetic effects of members of the TNF and TNFR superfamilies. Details are described in the text. EDA, ectodysplasin A; EDAR, EDA receptor.

et al., 1989). It is therefore not surprising that many molecular connections between TNF signalling and the cytoskeleton have since been described (Table 1). Some mechanisms by which TNFα might mediate cytoskeletal changes include the activation of the small GTPase RalA in the exocyst-dependent formation of filopodia (which relies on actin organization) (Sugihara et al., 2002), the activation of the myosin light chain kinase (Petrache et al., 2003a) and the regulation of the phosphorylation status of the adhesion-complex-associated actin-binding proteins of the ERM family (Koss et al., 2006), paxillin, focal adhesion kinase (FAK) and vinculin (Koukouritaki et al., 1999).

TNFα modulates the cytoskeleton via Rho-family GTPases

The Rho-family GTPases Cdc42, Rac and Rho (see Box 1), which are central to many signalling pathways that lead to cytoskeletal changes, have been widely implicated in TNFα-mediated cytoskeletal remodelling. For instance, treatment of fibroblasts with TNFα triggers the activation of Cdc42, which leads to filopodium formation and subsequent activation of Rac and Rho (Puls et al., 1999). In endothelial cells, TNFα-induced increases in F-actin levels, stress-fibre formation and cell contraction are associated with a hierarchical activation of Cdc42, Rac and Rho (McKenzie and Ridley, 2007; Neumann et al., 2002; Papakonstanti and Stournaras, 2004; Petrache et al., 2003b; Wojciak-Stothard et al., 1998).

There is thus ample evidence that Cdc42, Rac1 and RhoA act downstream of TNFα, but how a signal is transmitted from the TNFR to these small GTPases is not fully understood. It is not necessarily the case that the TNF receptor transduces the TNFα signal via one of the canonical adaptors that bind to the receptor's death domain (see above), because it has been proposed that the adaptor proteins TRADD, receptor-interacting protein (RIP) and TRAF2 do not participate in TNFα-induced activation of Rho GTPases (Puls et al., 1999). Thus, the effects on the actin cytoskeleton may be separable from the classical pathways downstream of the TNFR that involve caspase, JNK and NF-κB signals (Fig. 1). Furthermore, certain TNFα-dependent effects on the cytoskeleton do not require the receptor's death domain (Peppelenbosch et al., 1999), but instead require a more membrane-proximal region of the receptor, the neutral sphingomyelinase (N-SMase)-activating domain (Adam-Klages et al., 1996). One adaptor protein, factor associated with N-SMase activity (FAN), which binds to this region, is required for TNFα-dependent activation of Cdc42 and filopodium formation (Haubert et al., 2007). Other molecules that have been implicated in TNFα-stimulated modulation of GTPases include MAP kinases, phosphoinositide 3-kinases, PLCγl and ceramide (Hanna et al., 2001; Kutsuna et al., 2004; Papakonstanti and Stournaras, 2004).

It would be convenient if the Rho-family GTPases provided an example of a mechanism that distinguishes between TNFα-mediated apoptotic and non-apoptotic functions, but this is unfortunately not so. For example, TNF signalling can act through small GTPases not only to affect cell morphology, but also to deliver cell-death or cell-survival signals. In opossum kidney cells, the actin redistribution and polymerization that results from TNFα-mediated activation of Cdc42 can permit nuclear translocation of the anti-apoptotic NF-κB (Papakonstanti and Stournaras, 2004). Furthermore, TNFα-induced apoptosis can be suppressed by dominant-negative RhoA or by inhibition of the downstream target of RhoA, Rho-associated kinase (ROCK) (Petrache et al., 2003b). Moreover, Rac1 can contribute to TNFα-induced apoptosis in mammalian cell lines (Esteve et al., 1998).

Box 1. Regulating the activity of small GTPases
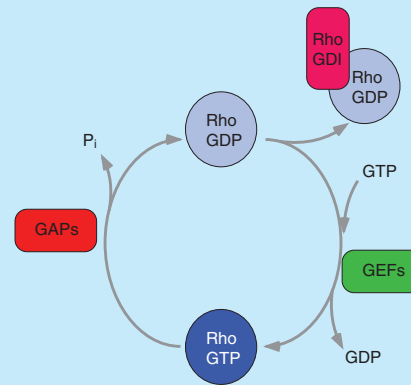
The many subclasses of small GTPases (such as Ras, Rho, Arf, Rab and Ran GTPases) participate in almost all cellular functions, including cell division, actin-cytoskeleton reorganization, cell adhesion, transport, gene expression, vesicle trafficking, growth and cell survival. The functions of small GTPases are regulated by conformational changes associated with shuttling between the active (or GTP-bound) state and inactive (or GDP-bound) state. Three classes of regulatory molecules are involved in controlling this cycling between GTP- and GDP-bound states. Guanine-nucleotide exchange factors (GEFs) activate small GTPases by promoting exchange of GDP for GTP. GTPase-activating proteins (GAPs) inactivate small GTPases by enhancing GTP hydrolysis to GDP. Guanine-nucleotide dissociation inhibitors (GDI) function as inhibitors by sequestering the GTPase in the GDP-bound inactive state, thereby preventing activation by GEFs.

Further interconnections between apoptotic and non-apoptotic mechanisms that involve the Rho GTPases exist at other levels. Beside its role as a downstream target of RhoA, ROCK can also be activated by caspases (Coleman et al., 2001; Sebbagh et al., 2001) and this might have a role in TNF-induced non-apoptotic cytoskeletal changes. Thus, the downstream signalling events that mediate cytoskeletal and other apoptotic changes are closely linked, and it will be important to tease apart these roles of TNF signalling.

The TNFR superfamily and the neuronal actin cytoskeleton

The growth and collapse of axonal growth cones presents a striking example of a morphogenetic function of TNFR-superfamily members. The path of growing axons is determined by the exploratory behaviour of the growth cone, which responds to chemo-attractive and repellent cues by growing, turning and retracting. These behaviours involve dramatic RhoA-dependent changes in the actin cytoskeleton (Gallo and Letourneau, 2004). Lack of RhoA activity allows growth, whereas activation of RhoA blocks growth. One set of molecules that regulate RhoA activity is made up of the members of the TNFR-superfamily p75 and TAJ (toxicity and JNK inducer, also known as TROY). Unlike TNFR1, which forms a homotrimeric complex, these molecules can each form a heterotrimeric complex with the transmembrane protein LINGO-1 and the GPI-linked Nogo receptor (NogoR) (Mi et al., 2004; Park et al., 2005; Shao et al., 2005; Wang et al., 2002) (Fig. 1). Neurite-growth-inducing p75 ligands, such as nerve growth factor (NGF) reduce RhoA activity, whereas myelin components that have

Table 1. Effects of TNFα on cytoskeleton and cell shape

Effects	Cell type	Molecular mediators	References
Permeability increase, cytoskeletal changes, changes in cell shape, increase in F-actin, formation of filopodia and membrane ruffles	Endothelial cells	ERM, p38 MAPK, PKC, Cdc42, Rac, Rho	(Goldblum et al., 1993; Kohno et al., 1993; Koss et al., 2006; McKenzie and Ridley, 2007; Triplett and Pavalko, 2006; Wojciak-Stothard et al., 1998)
Changes in cell shape and cytoskeletal organization, disruption of monolayer continuity	Endothelial cells	G-protein signalling	(Brett et al., 1989)
Chemotactic migration	Fibroblasts	–	(Postlethwaite and Seyer, 1990)
Formation of filopodia, lamellipodia and stress fibres	Fibroblasts	Cdc42, Rac1, RhoA	(Haubert et al., 2007; Puls et al., 1999)
Breakdown of stress fibres	Fibroblasts	–	(Scanlon et al., 1989)
Increased integrin expression and associated increase in cell attachment to type-1 collagen	Fibroblasts	–	(Ezoe and Horikoshi, 1993)
Modulation of adhesion and chemotaxis; actin depolymerization and morphological changes	Neutrophils	p38 MAPK, ERK	(Kutsuna et al., 2004; Lokuta and Huttenlocher, 2005)
Changes in cell shape and actin reorganization	Neutrophils	Integrins and cAMP	(Nathan and Sanchez, 1990)
Actin cytoskeleton reorganization	Epithelial cells	Paxillin and FAK	(Koukouritaki et al., 1999)
Enhanced cell migration	Epithelial cells	FAK	(Corredor et al., 2003)
Altered cell shape and cytoskeletal organization	Mesangial cells	Platelet activating factor, vinculin	(Camussi et al., 1990)
Modulation of migration and adhesion, tissue remodelling and wound closure	Keratinocytes	–	(Banno et al., 2004)

inhibitory effects on neurite outgrowth, such as myelin-associated glycoprotein (MAG) or Nogo, increase RhoA activity (Fig. 3).

One mechanism by which RhoA activity can be regulated is via the RhoGDP dissociation inhibitor (RhoGDI), which can bind to the intracellular domain of p75 (Yamashita and Tohyama, 2003). The binding of RhoGDI to p75 prevents the inhibition of RhoA by RhoGDI, allowing activation of RhoA by an as-yet-unknown GEF and subsequent growth-cone collapse (Park et al., 2005; Shao et al., 2005; Wang et al., 2002; Yamashita and Tohyama, 2003; Yamashita et al., 1999) (Fig. 3). This effect of p75 is stimulated by the growth inhibitors MAG or Nogo, but reduced by the growth inducer NGF. This situation therefore provides an example for the very direct effect of a TNFR superfamily member on a Rho GTPase.

TNFα and the TNFRs 1 and 2 can inhibit hippocampal neuron outgrowth and branching during inflammatory responses by activating RhoA (Neumann et al., 2002). However, unlike the immediate activation of RhoA that is caused by the p75 and TAJ (TROY) receptors, ligand-receptor binding in these cases leads to a delayed activation of RhoA (Neumann et al., 2002). As in the case of non-neuronal cells, a conserved theme emerges from these studies – various members of the TNF-receptor superfamily regulate morphogenesis by controlling RhoA activity, which in turn regulates the actin cytoskeleton.

One significant aspect of TNFα-mediated actin-cytoskeleton rearrangements in neurons, as well as other cells, is the absence of the induction of cell death. This is not due to an inherent inability of the neuronal receptors to recruit the apoptotic machinery, because the p75 receptor can induce apoptotic cell death in the mouse retina and spinal cord during development (Frade and Barde, 1999). The delivery of an apoptotic signal might be prevented by the recruitment of different sets of adaptors (for the apoptotic and non-apoptotic pathways) by the receptor, or by the divergence of the pathways at a later step. Alternatively, the apoptotic branch of the signalling cascade might be suppressed by NF-κB (reviewed by Karin and Lin, 2002; Muppidi et al., 2004), because p75 can also activate NF-κB (Carter et al., 1996), a prime target of TNFR1 signalling.

Other morphogenetic effectors of TNF

Other targets of TNF signalling that are closely linked with morphogenetic cell behaviour are the components of cell-adhesion complexes, which are assemblies of proteins that mediate adhesive contacts between cells and their environment (including other cells). Cell-adhesion complexes are dynamic, and can be modulated in their adhesiveness through the post-translational modification of their components. For example, TNFα treatment can increase the tyrosine phosphorylation of several adherens-junction proteins (including vascular endothelial cadherin, catenins and integrins) and this modification is mediated either by the Src-family kinases (Angelini et al., 2006; Bouaouina et al., 2004) or by JNK (Nwariaku et al., 2004). Protein kinase C α (PKCα) or reactive oxygen species (ROS) might mediate Src activation downstream of TNFα (Huang et al., 2003; Tiruppathi et al., 2001). In one instance, in bone marrow macrophages, TNFα has been shown to induce Src autophosphorylation and increase its kinase activity (Abu-Amer et al., 1998). In endothelial cells, TNF-mediated modulation of tight-junction proteins is correlated with changes in cell shape (McKenzie and Ridley, 2007). Furthermore, recent reports show that integrin ligation and cell-matrix interaction can modulate TNFα-induced apoptosis independently of NF-κB activation, which demonstrates a crosstalk between integrin and TNF signalling (Bieler et al., 2007; Chen et al., 2007). Depending on the context, tyrosine phosphorylation of components of adherens junctions can lead to stabilization or to internalization of junctions. Whereas internalization can form part of an apoptotic response, such as the extrusion of dying cells from coherent cellular aggregates, stabilization of junctions is more likely to be involved in the non-apoptotic functions of TNF signalling.

Morphogenetic functions of TRAFs

Many TNF-dependent signals are transmitted by TRAFs (reviewed by Aggarwal, 2003; Wajant et al., 2003). Some TRAFs, in addition to acting downstream of TNFRs in the apoptotic and inflammatory responses, may also participate in pathways that are dedicated purely
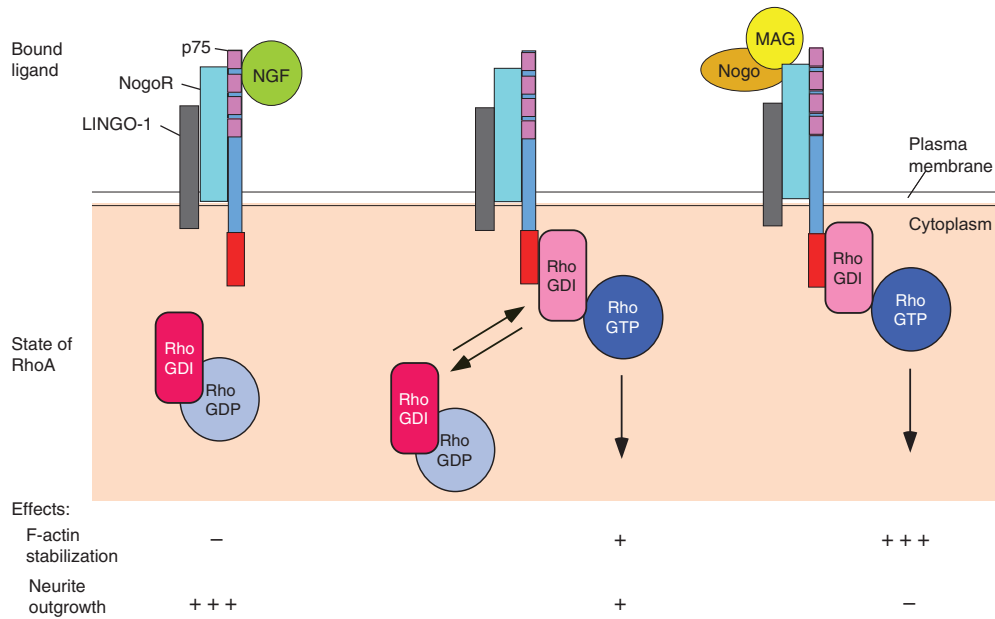
Fig. 3. Model for signalling from p75–NogoR–LINGO-1 to RhoA. In neuronal cells, binding of NGF to the p75–NogoR–Lingo-1 receptor complex inhibits RhoA activity (left), whereas the binding of MAG or Nogo has the opposite effect (right). When the heterotrimeric receptor complex is not bound to any extracellular ligands (centre) it interacts weakly with a complex of RhoGDI and RhoA. Binding of the p75 cytoplasmic tail by the RhoGDI-RhoA complex inhibits the activity of RhoGDI; consequently, RhoA is active (dark blue). When the ligand NGF binds to p75 (left), the interaction of p75 with the RhoGDI-RhoA complex is disrupted, RhoGDI is no longer inhibited, exchange of GDP for GTP cannot occur and RhoA is inactive (light blue). This acts as a permissive signal to the actin cytoskeleton, facilitating neurite outgrowth. By contrast, when the ligands Nogo or MAG bind to NogoR or LINGO-1 (right), the interaction of p75 with the RhoGDI-RhoA complex is strengthened, causing a strong inhibition of RhoGDI, which permits GDP-GTP exchange and an increase in the level of RhoGTP. This leads to actin stabilization and concomitant inhibition of neurite outgrowth.

to morphogenetic and developmental functions. For instance, TRAF6 has been shown to have a dedicated role in ectodermal organ morphogenesis and development (Ohazama et al., 2004), and TRAF4 in cell polarity and embryonic development (Cherfils-Vicini et al., 2008; Kedinger et al., 2008; Regnier et al., 2002; Shiels et al., 2000). It appears that TRAF4 is associated preferentially or exclusively with morphogenetic events. In addition to its role in embryonic development and neurulation (Regnier et al., 2002), TRAF4 is also needed for the efficient migration of dendritic cells (Cherfils-Vicini et al., 2008). A recent study shows that TRAF4 localizes to tight junctions in epithelial cells and is crucial for epithelial homeostasis (Kedinger et al., 2008). TRAF4 has so far not been shown to interact physically with receptors of the TNFR superfamily, in spite of its high homology to the other TRAFs. However, TRAF4 can signal to JNK as well as to NF-κB, and situations have been discovered in which it is involved in signal transmission downstream of TNF-superfamily receptors (Esparza and Arch, 2004; Fleckenstein et al., 2003; Ye et al., 1999). In view of these connections, we consider it useful to discuss the role of TRAF4 in the context of TNF-dependent cell-shape changes.

TRAF4 mediates cell polarity
Recent work has demonstrated a role in *Drosophila melanogaster* for TRAF4 (previously named TRAF1) and the TNF homologue Eiger in the polar distribution of cell-fate determinants during asymmetric cell division of neuroblasts (Wang et al., 2006). TRAF4 localizes asymmetrically to the apical cell cortex of dividing neuroblasts, where it interacts with membrane-associated Par3, and this localization depends on signalling by Eiger. Asymmetric cell division was compromised in the absence of TNF or TRAF4 (Wang

et al., 2006). This is especially interesting in view of evidence for crosstalk between polarity proteins and Rho GTPases, such as during asymmetric cell division or in neuronal cell polarity (Iden and Collard, 2008; Mertens et al., 2006; Solecki et al., 2006). It is therefore a reasonable hypothesis that the Rho-family GTPases also have an important role in the case of TNF-dependent asymmetric cell division.

TRAF4 mediates subcellular localization of ROS production
In mammals, TRAF4 is involved in a different morphogenetic signalling pathway downstream of TNF that involves targeting the production of ROS to focal adhesions in lamellipodia and membrane ruffles at the leading edge; this targeted production promotes cell migration. ROS have been implicated downstream of TNF in diverse contexts, including the activation of the known mediators of TNF signalling, NF-κB (Zhang and Chen, 2004) and JNK (Kamata et al., 2005), and the induction of necrosis (Kim et al., 2007). Several mechanisms can induce ROS production, and these correlate with different physiological effects.

Mitochondria-derived ROS are mainly implicated in cell death, whereas ROS production by NADPH oxidase 2 (Nox2) is best known for its function in granulocytes as an anti-bacterial defence mechanism (Lambeth, 2004). However, ROS production by Nox2 and other isoforms of NADPH oxidase, such as Nox1, Nox3 and Nox4 has also been associated with cytoskeletal rearrangements that lead to cell migration and other cellular responses. It is not clear how the source of ROS production confers signalling specificity, although the type of NADPH oxidase and site of production are likely to be important factors, as ROS are short-lived molecules (for a review, see Bedard and Krause, 2007).
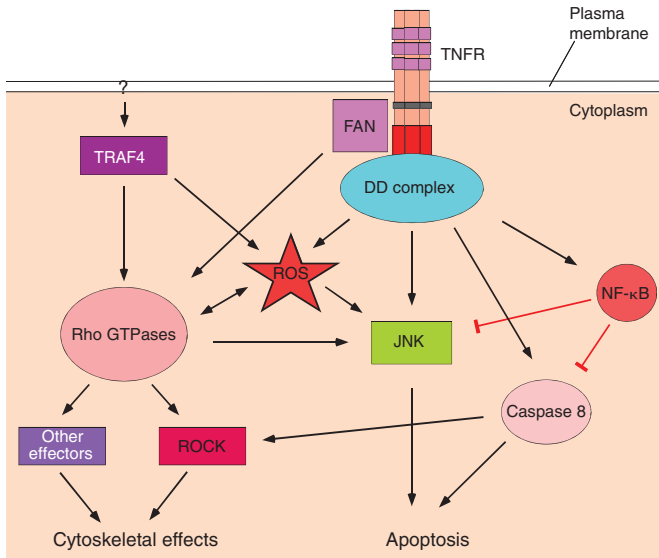
Fig. 4. Possible pathways from TNFRs to modifications of the cytoskeleton. The death-domain complex (DD complex) comprises the signalling adaptors that bind to the death domain of TNFRs. It is unknown whether TRAF4 is activated by a TNFR. Caspases and JNK signalling are the major apoptotic targets of TNFRs, whereas NF-κB is the transcription factor that regulates cell survival downstream of TNFRs. The Rho GTPase family of cytoskeletal regulators forms a convergence point for diverse modes of TNF signalling and signalling intermediates, including FAN, ROS, JNK and TRAF4. Caspase 8 can act on ROCK and might therefore modulate the cytoskeleton independently of Rho GTPases. Rho GTPases exert their effect on the cytoskeleton through several effectors, including ROCK. The cytoskeletal changes might be promoted at different levels of the TNF signalling pathway, whereas the TNF-dependent apoptotic pathway might at the same time be suppressed by NF-κB, thereby allowing morphogenesis to occur independently of cell death. The arrows do not necessarily reflect direct interactions and are a subset of the possible mechanisms by which TNF signalling can lead to morphogenetic effects.

Similar to the pathways described above, the morphogenetic processes that involve ROS production downstream of TNF signalling are mediated by a Rho-family GTPase, in this case Rac. Rac is required for the production of ROS by Nox2 in TNFα-stimulated endothelial cells, and both Rac and Nox2 are needed for endothelial-cell migration (Van Buul et al., 2005). When expressed as GFP-fusion proteins in human umbilical-vein endothelial cells (HUVECs), the Nox2 subunits p47phox and p67phox show a striking TNFα-dependent translocation to the tips of TNFα-induced membrane ruffles together with Rac (Van Buul et al., 2005). The localization and activation of Nox2 at membrane ruffles is brought about by the binding of its subunit p47phox to TRAF4, which in turn is recruited to focal-adhesion complexes by the paxillin paralogue Hic5 (Wu et al., 2005). Disruption of the active complex between TRAF4 and Hic5 at focal adhesions, by loss of either TRAF4 or Hic5, abolishes directed cell migration (Wu et al., 2005). Given the many apparent links between processes that involve TRAF4 and those that are controlled by TNF signalling, it is possible that a direct molecular connection between TRAF4 and the TNFR complex will yet be found. Alternatively, such a connection may have been lost during evolution as TRAF4 became specialized for other functions, or (if TRAFs pre-date the use of TNF signalling as it now exists) there may never have been a connection.

A morphogenetic signalling pathway?

The complete transduction cascade by which TNF signalling directs morphogenetic events has not been determined in any of the situations we have discussed, but the participation of the Rho-family GTPases features as a recurrent theme. As the pathway to cytoskeletal changes is also needed during apoptotic and inflammatory responses, it is likely that many components are shared. Nevertheless, it must be possible to uncouple the full apoptotic response from one that involves only the cytoskeleton. We can envisage various points at which the signalling pathway to the cytoskeleton might be uncoupled from the death-inducing pathways (Fig. 4).

All cells can in principle undergo apoptosis but, in many of the cases described above, the response to TNF signalling is not apoptosis; thus, there must be mechanisms that allow cells to direct the TNF signal in one or the other direction (towards apoptosis or towards cytoskeletal changes), irrespective of the level at which the signal to the cytoskeleton diverges. The same problem is faced in the decision between death and inflammatory responses downstream of TNF signalling, and models for this have been proposed. Signalling is usually associated with the activation of NF-κB, which, with its ability to block steps of the apoptotic pathway, might be a central factor in determining which pathway predominates (Karin and Lin, 2002; Muppidi et al., 2004).

The first point at which a specific link between TNF and the Rho GTPases and the cytoskeleton might be made is during the recruitment of adaptors to the TNF receptor. Although the connection between p75 and RhoA is well understood (see above), it remains to be discovered how other TNFRs link to small GTPases. As discussed above, death-domain-binding adaptors are not necessarily required to mediate Cdc42 activation and cytoskeletal changes (Puls et al., 1999), and molecules such as FAN that transmit signals from the TNFR to the cytoskeleton and bind to other parts of the intracellular domain of the TNFR are now being identified (Haubert et al., 2007) (Fig. 4).

It is probable, however, that an uncoupling of the pathway to cytoskeletal change can also occur further downstream. A significant feature of TNFα-induced cell death and cytoskeletal changes is the role that is fulfilled by the downstream effectors JNK and NF-κB. Their functions in the regulation of cell death and cell shape and their interaction with the Rho-family GTPases appear to be closely intertwined. Rac1 and Cdc42 can activate JNK and NF-κB and are, at least in some situations, required for TNFα-dependent regulation of JNK and NF-κB (Minden et al., 1995; Perona et al., 1997; Williams et al., 2008). Conversely, JNK is well established as a mediator of cell-shape changes (reviewed by Xia and Karin, 2004), and it is therefore conceivable that effects of TNFα on the cytoskeleton are signalled through JNK. Finally, even components that are normally considered to be committed to apoptotic signalling, such as the caspases, also have non-apoptotic functions (Kanuka et al., 2005; Kuranaga et al., 2006).

It is theoretically possible that the signal received by the TNF receptor in cells in which a non-apoptotic outcome is desired never even triggers the TRADD-mediated recruitment of FAS-associated death domain (FADD) and caspase-8 as a first step of signalling cascades that produce apoptotic signals. However, death-domain adaptors are present in all cells, so this is difficult to imagine. Indeed, recent data show that the signalling outcome might be affected by whether TNFR1 partitions into lipid-raft microdomains (reviewed by Muppidi et al., 2004). It is probable that, in most instances, the apoptotic signal-transduction cascade is initiated, but not followed

to completion. Just as NF-κB has been suggested to block the JNK pathway and caspase activation (Karin and Lin, 2002; Muppidi et al., 2004), thereby allowing cell-survival signals to be effective, so it could block cell death in cases in which only cytoskeleton-regulating signals are desired (Fig. 4). Other ways of balancing the alternative signals might be by regulating the levels of JNK, NF-κB or other modulators (such as caspases) by differences in signalling thresholds for apoptotic and morphogenetic responses, or by co-stimulation of other pathways.

Conclusions and perspectives

In this Commentary, we have drawn attention to situations in which TNF signalling has been found to have morphogenetic functions that are not necessarily associated with cell death or inflammation. Many of the signalling components that promote these functions are the same as those that are used in morphogenetic processes during apoptotic and inflammatory responses, with the Rho GTPases occupying a particularly prominent position. It is worth noting that the unicellular organism *Dictyostelium discoideum*, which does not undergo receptor-mediated caspase-dependent apoptosis, nevertheless has proteins that are homologous to components of the TNF signalling pathway. Thus it has a homologue of TNFR-associated protein 1 (TRAP1), which colocalizes with actin, as well as a TRAF-like protein (Morita et al., 2002; Morita et al., 2004). This might suggest that an ancient morphogenetic function of these components pre-dates their use in inflammatory and apoptotic functions in multicellular organisms. The TNF pathway might therefore have recruited an existing cytoskeletal-control machinery to regulate part of the apoptotic and inflammatory programme in multicellular animals. The fact that members of the different TNF- and TNFR-superfamily signalling pathways share the ability to control morphogenetic cell behaviour further supports the notion that this is an ancient function that has been conserved from a common ancestral signalling cassette.

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